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EXHIBIT A

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Improvement of Hepatitis B Virus DNA Vaccines by Plasmids Coexpressing Hepatitis B Surface Antigen and Interleukin-2

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DNA vaccines encoding a viral protein have been shown to induce antiviral immune responses and provide protection against subsequent viral challenge. In this study, we show that the efficacy of a DNA vaccine can be greatly improved by simultaneous expression of interleukin-2 (IL-2). Plasmid vectors encoding the major (S) or middle (pre-S2 plus S) envelope proteins of hepatitis B virus (HBV) were constructed and compared for their potential to induce hepatitis B surface antigen (HBsAg)-specific immune responses with a vector encoding the middle envelope and IL-2 fusion protein or with a bicistronic vector separately encoding the middle envelope protein and IL-2. Following transfection of cells in culture with these HBV plasmid vectors, we found that the encoded major protein was secreted while the middle protein and the fusion protein were retained on the cell membrane. Despite differences in localization of the encoded antigens, plasmids encoding the major or middle proteins gave similar antibody and T-cell proliferative responses in the vaccinated animals. The use of plasmids coexpressing IL-2 and the envelope protein in the fusion or nonfusion context resulted in enhanced humoral and cellular immune responses. In addition, the vaccine efficacy in terms of dosage used in immunization was increased at least 100-fold by coexpression of IL-2. We also found that DNA vaccines coexpressing IL-2 help overcome major histocompatibility complex-linked nonresponsiveness to HBsAg vaccination. The immune responses elicited by HBV DNA vaccines were also modulated by coexpression of IL-2. When restimulated with antigen in vitro, splenocytes from mice that received plasmids coexpressing IL-2 and the envelope protein produced much stronger T helper 1 (Th1)-like responses than did those from mice that had been given injections of plasmids encoding the envelope protein alone. Coexpression of IL-2 also increased the Th2-like responses, although the increment was much less significant.

Recently, a new technology, in which recombinant genes in the form of a plasmid expression vector can be delivered and expressed in animal muscle, has been described (50, 51). Based on this technique, a novel type of vaccine, termed nucleic acid vaccine or DNA vaccine with DNA instead of proteins in the vaccine formulation has been developed (9, 26). The expression vectors used for DNA vaccines contain the gene(s) for an antigenic portion of a virus, such as the core protein or the envelope protein, usually under the transcriptional control of a viral promoter. Direct injection of the DNA into skeletal muscles results in the synthesis of viral proteins in the host and thus may mimic the action of attenuated vaccines. The in vivo-synthesized viral protein can enter both the major histocompatibility complex (MHC) class I and class II antigen-processing pathways to activate specific immunization. Animal models in which DNA vaccines induce a broad range of immune responses, including antibodies, cytotoxic T cells, T-cell proliferation, and protection against challenge with the pathogen, have been reported (21–24, 28, 40, 41, 45, 46, 53, 56, 57).

Despite the initial success of this approach to genetic vaccination, it has been clearly demonstrated from recent reports that some DNA vaccines elicit much stronger immune response than others. With one single intramuscular injection of plasmids encoding influenza virus nucleoprotein (37) or HBsAg (28), the immunized animals developed long-lasting immunity. By contrast, plasmids encoding the nucleoprotein of lymphocytic choriomeningitis virus (25, 57), glycoprotein gp14 of equine herpesvirus 1 (34), or paramyosin Sj97 of the parasite *Schistosoma japonicum* (55) were much less effective, even after repeated injection. The mechanisms underlying the different efficacies of various DNA vaccines have not yet been clearly addressed but presumably are related to the efficacy of transfection, the expression and antigenic nature of the encoded antigen, and the ability of the protein to be appropriately presented to the immune system.

The use of cytokines as immunological adjuvants can enhance various immune responses when administered during the development of an immune response to a particular antigen (8, 15). Interleukin-2 (IL-2) is perhaps the most extensively studied of all cytokine adjuvants. When administered as multiple injections following the antigen, IL-2 enhances the protection against challenge with the infectious agent (3, 33, 49). When used in appropriate adjuvant preparations, IL-2 has been reported to overcome MHC-linked nonresponsiveness to peptide antigens (12, 20) or to reverse the nonresponsiveness to HBsAg vaccination in immunocompromised patients (27). The adjuvant efficacy was further enhanced by physical linkage of IL-2 and antigen to ensure the cytokine effect on the local environment where immune responses occurred. Fusion proteins consisting of IL-2 with herpes simplex virus glycoprotein D (13, 14) or a tumor idiotypic protein (5) have been shown to enhance immunity to the conjugated antigen and provide protection against subsequent challenge.

In this study, we used plasmid vectors encoding hepatitis B virus (HBV) envelope protein as a model to study how DNA vaccines may be modulated by the simultaneous expression of IL-2. The structural gene encoding HBV envelope proteins terminates in a single stop codon but can initiate at three possible translational start codons, which define the pre-S1

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(residues 1 to 119), pre-S2 (residues 120 to 174), and S (residues 175 to 400) coding regions, yielding the large (pre-S1 plus pre-S2 plus S), middle (pre-S2 plus S), and major (S) envelope proteins, respectively (11, 44). To ensure that IL-2 was delivered at the sites of immune interactions, we constructed a plasmid encoding HBV middle envelope and IL-2 fusion protein and a bicistronic plasmid encoding the envelope protein and IL-2 separately and compared their efficacy in inducing immune responses to plasmids encoding the HBV envelope protein alone. We found that plasmids coexpressing IL-2 activities had an increased vaccine efficacy by at least 100-fold and induced much stronger humoral and cellular immunities to HBsAg.

MATERIALS AND METHODS

Construction of expression vectors. The major and middle envelope genes of HBV were amplified by PCR from plasmid pEco63 (ATCC 31518; American Type Culture Collection, Rockville, Md.), which contains the full-length HBV genome, and cloned into the *Bam*HI/*Eco*RI sites in the polylinker region of plasmid pcDNA3 (Invitrogen, San Diego, Calif.) to produce plasmids pS and pS2-S (see Fig. 1). This eukaryotic expression vector contains the cytomegalovirus early promoter/enhancer sequence and the polyadenylation and 3′-splicing signals from bovine growth hormone. The upstream primers for the major and middle envelope genes contain a *Bam*HI site and the ATG start codon of the S and pre-S2 sequences, respectively. The downstream primer contains an *Eco*RI site 3′ to the stop codon and a *Hin*dIII site inserted between the last codon and the stop codon of the S gene to facilitate the following construction of plasmids encoding the middle envelope and IL-2 fusion molecules. The fragment containing mature mouse IL-2 sequence was generated by PCR from plasmid pmur-1 (ATCC 37553). The upstream primer contains two Gly codons 3′ to the *Hin*dIII site, and the downstream primer contains a stop codon 5′ to the *Eco*RI site. PCR products were digested with *Hin*dIII and *Eco*RI, gel purified, and inserted between the *Hin*dIII and *Eco*RI sites of plasmid pS2-S to produce pS2-S-IL2 (Fig. 1). A bicistronic plasmid designated pS2-S/pIL2 (Fig. 1), encoding the HBV middle envelope protein and IL-2 under the control of discrete cytomegalovirus promoters and bovine growth hormone polyadenylation sequences, was generated by replacing the *Bgl*II/*Eco*RI immunoglobulin Cκ gene and the *Sal*I/*Bam*HI Cγ1 gene of plasmid vector pTCAE (provided by M. Reff, IDEC Corp., San Diego, Calif.) with the PCR-amplified pre-S2-S and mouse IL-2 fragments, respectively. Plasmid DNA was purified from transformed *Escherichia coli* DH5α by CsCl gradient ultracentrifugation and stored as pellets at −70°C. For experimental use, the DNA was reconstituted in sterile saline at 1 mg/ml. All samples were tested by the *Limulus* amebocyte lysate assay (Sigma, St. Louis, Mo.) to ensure that they were free of endotoxin contamination.

Cell transfection and plasmid gene expression. C2C12 mouse myoblasts (10^5 cells; ATCC 1772) were cultured in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum (FBS) in a six-well tissue culture plate until the cells reached approximately 50 to 80% confluence. Plasmid DNA transfection was performed with Lipofectamine (Gibco BRL, Gaithersburg, Md.) as specified by the manufacturer. Briefly, 3 μg of plasmid DNA was mixed with 20 μl of Lipofectamine in 200 μl of OPTI-MEM medium (Gibco BRL) at room temperature. Following a 20-min incubation, the DNA-liposome complexes were diluted in 800 μl of OPTI-MEM and slowly added to cells which had been prewashed twice with 5 ml of OPTI-MEM. After a 16-h incubation, the DNA-liposome complexes were removed, 2 ml of complete growth medium was added to each well, and the incubation was continued for another 48 h. The supernatant was collected and stored at −80°C for later analysis. The transfected cells were washed twice with cold phosphate-buffered saline (PBS), lysed by addition of 300 μl of Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 2 mM EDTA, 50 mM Tris [pH 7.6]), and incubated on ice for 20 min. Following centrifugation at 10,000 × *g* for 10 min at 4°C, the lysate was collected for analysis.

Immunoblot assays were performed as described previously (5) with some modifications. In brief, sample proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE 12.5% polyacrylamide) and transferred to nitrocellulose in 25 mM sodium phosphate buffer (pH 7) for 2 h at 180 mA. The filters were first treated with blocking buffer (5% powdered milk in PBS) for 1 h and then incubated overnight with mouse polyclonal anti-HBsAg antiserum (1:100) or with rat anti-mouse IL-2 monoclonal antibody MAb (1:500; JES6-1A12; immunoglobulin G2a; IgG2a; Pharmingen, San Diego, Calif.). The polyclonal anti-HBsAg antiserum was made by pooling sera from five mice that had been given intraperitoneal injections of 2 μg of recombinant HBsAg with complete Freund's adjuvant and bled 4 weeks after immunization. Following a wash in PBS-Tween (0.05%), the membranes were incubated for 1 h with goat anti-mouse IgG-horseradish peroxidase (HRP) (1:2,000; Cappel, Organon Teknika, Veedijk, Belgium) or goat anti-rat IgG-HRP (1:1,000; Cappel) in blocking buffer. After 3 10-min washings in PBS-Tween, blots were developed by the enhanced chemiluminescence Western blot detection system (Amersham, Little Chalfont, United Kingdom) and exposed to X-ray film.

A commercial enzyme-linked immunosorbent assay (ELISA) kit (SURASE; General Biologicals Co., Hsinchu, Taiwan) was used to analyze the expression of HBsAg by transfected cells. Briefly, 50 μl of diluted samples and 50 μl of HRP-conjugated guinea pig polyclonal anti-HBsAg antibodies were added to each well of a 96-well ELISA plate coated with an HBsAg-specific mouse MAb and incubated at 40°C for 3 h. After the plate was washed with PBS-Tween buffer, 100 μl of OPD solution (3 mg of *o*-phenylenediamine in citrate-phosphate buffer containing 0.02% H_2O_2) was added to each well and the plate was incubated at room temperature for 30 min. The reaction was stopped by adding 100 μl of 2 N H_2SO_4, and the absorbance at 492 nm was measured on an ELISA reader (Thermomax; Molecular Devices, Menlo Park, Calif.). The amount of HBsAg in each sample was calculated from a standard curve generated by using positive control HBsAg provided in the kit. All results are presented as the mean ± standard deviation (SD).

The IL-2 bioactivity of test samples was assayed by a proliferative assay as described previously (5). In brief, samples were added in triplicate to 96-well plates in complete RPMI 1640 plus 10% FBS with 5,000 IL-2-responsive HT-2 cells (48) to a total volume of 0.1 ml and incubated for 16 to 24 h at 37°C under 5% CO_2 in a humidified incubator. Then 1 μCi of [^{3}H]thymidine (Amersham) was added to each well in 50 μl of growth medium, the cells were harvested 4 to 6 h later with a FilterMate (Packard, Meriden, Conn.) automatic cell harvester, and incorporated radioactivity was determined with a TopCount microplate scintillation counter (Packard). Recombinant murine IL-2 (Genzyme, Cambridge, Mass.) was included in the assay as a positive control. All results are presented as the mean counts per minute ± SD.

Immunization of mice. Female BALB/c (*H-2*^{d}) and C57BL/6 (*H-2*^{b}) mice were purchased from the Laboratory Animal Facility, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan. Female C3H/HeN (*H-2*^{k}) mice were obtained from the Animal Facility, National Cheng Kung University, Tainan, Taiwan. Female congenic B10.M (*H-2*^{f}) mice were purchased from Harlan UK Limited, Bicester, Oxon, United Kingdom. Mice were housed at the Laboratory Animal Facility, Institute of Biomedical Sciences, Academia Sinica. All mice were immunized at 6 to 8 weeks of age as described previously (51). In brief, groups of five mice were anesthetized and given intramuscular injections, into the left quadriceps, of different doses (100, 10, or 1 μg) of plasmid DNA dissolved in 100 μl of normal saline. In experiments with B10.M mice, each animal was given an injection of 100 μg of DNA and boosted with an additional dose 14 weeks after the first immunization. For mice immunized with recombinant HBsAg vaccines, a yeast-derived vaccine, H-B-Vax II (Merck Sharp & Dohme, West Point, Pa.) containing 10 μg of HBsAg per ml adsorbed onto aluminum hydroxide, was used. Each animal was given an intraperitoneal injection of 4 μg of proteins and boosted with the same dose at weeks 3 and 14.

Antibody assay. Serum samples were collected by tail bleeding at different times beginning 1 week after immunization and analyzed for the presence of HBsAg-specific antibodies. Microtiter plates were coated with 100 μl (5 μg/ml) of yeast-derived recombinant HBsAg per ml. After incubation with 200 μl of 5% powdered milk in PBS on each well for 1 h to prevent nonspecific binding, 50 μl of serial dilutions of test sera was added to each well and incubated overnight at 4°C. After the samples were washed with PBS, bound proteins were detected with HRP-conjugated goat anti-mouse IgM (1:2,000; Cappel) or anti-mouse IgG (1:2,000; Cappel). Color was generated by adding 2,2′-azino-bis(ethylbenzthiazoline sulfonic acid), and the absorbance at 492 nm was measured on an ELISA reader. For measurement of IgM anti-HBs antibodies, readings were referenced to a standard serum pooled from four mice given intraperitoneal injections of 2 μg of recombinant HBsAg with complete Freund's adjuvant and bled 4 weeks after immunization. The standard serum was calculated as having a reading of 100 ELISA units/ml. For analysis of IgG anti-HBs antibodies, a control mouse IgG MAb (H25B10; ATCC CRL-8017) was used as the reference. Concentrations of HBsAg-specific antibodies in serum samples were estimated from the standard curve and expressed as units per milliliter for IgM and micrograms per milliliter for IgG, respectively. The total anti-HBs antibodies were quantitated by using two different commercial kits, the ANTISURASE kit (General Biologicals Co.) and the AUSAB-EIA kit (Abbott Laboratories, North Chicago, Ill.). Assays were performed as specified by the manufacturer. The amount of anti-HBs antibody was calculated from a standard curve generated by using the anti-HBs positive control provided in the ANTISURASE kit or the AUSAB quantitation panel (Abbott Laboratories), in which the anti-HBs concentrations were established with the World Health Organization hepatitis B Ig reference preparation and expressed as milli-international units per milliliter.

Lymphocyte proliferative assay. To determine if the plasmid DNA immunization could induce HBsAg-specific lymphoproliferative responses, C57BL/6 mice were immunized by injection of 100, 10, or 1 μg of different HBV plasmid vectors in the quadriceps muscle. On day 14, immune splenocytes were collected for the proliferative assay. CD4-depleted and CD8-depleted immune splenocytes were prepared by immunomagnetic depletion of CD4$^+$ and CD8$^+$ lymphocytes, respectively, and replaced with the same number of purified CD4$^+$ or CD8$^+$ cells isolated from naive mice. The immunomagnetic depletion or enrichment of CD4$^+$ and CD8$^+$ lymphocytes was performed by magnetic activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) with microbeads conjugated with rat anti-mouse CD4 and CD8 MAb (Miltenyi Biotec), respectively, as specified by the manufacturer. To perform the lymphoproliferative assay, 100 μl of 2 × 10^6 unfractionated, CD4-depleted, or CD8-depleted splenocytes per ml in

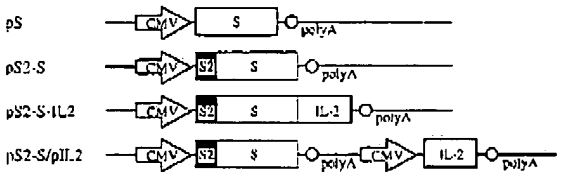

FIG. 1. Schematic diagram of the HBV envelope protein expression vectors. The transcriptional control sequences are shown: CMV, cytomegalovirus early promoter/enhancer sequence; polyA, bovine growth hormone termination and processing signals; S2 and S, HBV pre-S2 and S sequences, respectively. In plasmid pS2-S-IL2, a mature mouse IL-2 fragment was separated from the S gene by a short linker sequence encoding Lys-Leu-Gly-Gly. The IL-2 gene contained in the bicistronic vector, pS2-S/pIL2, represents the full-length cDNA sequence of mouse IL-2 including the signal sequence.

complete RPMI 1640 plus 10% FBS was added to each well in 96-well flat-bottomed plates. Stimulated wells received purified recombinant HBsAg at 30 or 10 μg/ml; transferrin (120 μg/ml; Sigma) served as a negative control antigen, and concanavalin A (5 μg/ml; Pierce, Rockford, Ill.) served as a positive mitogenic control. Control wells received cells only. Cells in all the wells were cultured in a total volume of 200 μl of medium. After 4 days in culture, the cells were pulsed with [^{3}H]thymidine (1 μCi/well) for 18 h and harvested with FilterMate (Packard), and incorporated radioactivity was determined by using TopCount (Packard). The stimulation index was calculated as the mean counts per minute of the stimulated wells divided by the mean counts per minute of the control wells.

Cytokine release assays. Splenocytes were cultured as described above with the same panel of antigens or mitogen over the same range of concentrations, except that after 3 days in culture, cell-free supernatants were harvested and assayed immediately or stored at −80°C. These supernatants were screened for the presence of gamma interferon (IFN-γ), IL-2, and IL-4 with an ELISA detection system (Pharmingen). The capture antibodies for murine IFN-γ, IL-2, and IL-4 were R46A2 (rat IgG1), JES6-1A12 (rat IgG2a), and BVD4-1D11 (rat IgG2b), respectively. The detection biotinylated antibodies for IFN-γ, IL-2, and IL-4 were XMG1.2 (rat IgG1), JES6-5H4 (rat IgG2b), and BVD6-24G2 (rat IgG1), respectively. Briefly, microtiter plates were coated overnight at 4°C with 50 μl of anti-cytokine capture MAb at 2 μg/ml. The plates were washed twice with PBS-Tween and blocked with 200 μl of 10% bovine calf serum in PBS per well for 2 h at room temperature. Then the plates were washed twice and incubated with duplicates of serially diluted samples and standards overnight at 4°C. Then 100 μl of the biotinylated anticytokine MAb at 2 μg/ml was added to each well, and the mixture was incubated at room temperature for 1 h. The plates were then washed six times, 100 μl of avidin-phosphatase (1/500 dilution; Pharmingen) was added, and the mixture was incubated at room temperature for 30 min. Following multiple final washings, the color was developed with *p*-nitrophenyl phosphate (Sigma) as the substrate, and the absorbance at 405 nm was measured with an ELISA plate reader. The concentration of cytokines in the samples was determined from the standard curve.

RESULTS

Construction and characterization of expression vectors. IL-2 has long been known to be a B- and T-cell growth factor and is capable of enhancing immune responses when administered in conjunction with a particular antigen (3, 33, 49). To test for an effect of IL-2 on the immune responses to a DNA vaccine, we constructed plasmids encoding HBV envelope proteins as a model system (Fig. 1). The structural genes encoding the major (S) and middle (pre-S2 plus S) HBV envelope proteins were placed in a cukaryotic expression vector under the transcriptional control of cytomegalovirus promoter/enhancer to make plasmids pS and pS2-S, respectively. A genetic fragment encoding mature murine IL-2 was inserted right after the 3′ end of the HBV S gene in pS2-S to make plasmid pS2-S-IL2, which was expected to make a middle envelope–IL-2 fusion protein separated by a 4-amino-acid linker (Lys-Leu-Gly-Gly). We also made a bicistronic vector, pS2-S/pIL2, to express HBV middle envelope protein and IL-2 within the same construct under discrete transcriptional control.

Mouse C2C12 myoblasts were transiently transfected with each of the HBV envelope protein expression vectors, with the parental plasmid, pcDNA3, serving as a negative control. At 2 days after transfection, the HBsAg and IL-2 molecules present in the culture supernatant or those that remained associated with cells were analyzed by immunoblotting techniques. Figure 2A shows the analysis of cellular fraction of the transfected cells. Proteins encoded by pS, pS2-S, pS2-S-IL2, and pS2-S/pIL2 but not by the control plasmid were recognized by specific anti-HBs antibodies. Plasmids pS2-S and the bicistronic vector pS2-S/pIL2 produced the expected middle envelope protein, which migrated slower on SDS-PAGE than did the major envelope protein encoded by plasmid pS. Plasmid pS2-S-IL2 encoded an HBV envelope protein which migrated slowest on the gel, indicating that it contained the IL-2 tail. This was further confirmed by the result that this HBV envelope fusion protein was specifically recognized by an anti-IL-2 MAb (Fig. 2A). The intracellular IL-2 synthesized by pS2-S/pIL2 was not detected by immunoblotting since most of the IL-2 molecules were secreted (see below). We did not detect any major envelope protein in cells transfected with plasmid pS2-S, pS2-S-IL2, or pS2-S/pIL2, indicating that translation of envelope proteins by these plasmids was initiated mainly from the start codon of the pre-S2 region. We also performed the immunoblot analysis on culture supernatant and found that only cells transfected with pS, but not the pre-S2 region-containing plasmids, secreted the envelope protein. This result indicated that the 55-amino-acid pre-S2 peptide redirected a secreted major envelope protein to be retained in the cells. Using flow cytometry analysis and immunofluorescence microscopy, we have shown that the middle envelope protein and the IL-2-containing fusion protein were expressed mainly on the cell surface (data not shown). To further confirm the cellular localization of the various HBV envelope proteins, a commercial ELISA kit which can detect HBsAg concentrations as low as 0.1 ng/ml was used. As shown in Fig. 2B, all recombinant vectors except the control plasmid produced similar levels of HBsAg in the cellular fraction. Again, in the culture supernatant, no HBsAg was detected from cells transfected with the pre-S2 region-containing plasmids, while cells transfected with plasmid pS secreted significant amounts of HBsAg.

To determine the IL-2 activity of proteins encoded by the different plasmid vectors, samples of transfected cells were analyzed for their ability to support the proliferation of a murine IL-2-responsive T-cell line, HT-2 (48). Supernatant and cellular fraction obtained 2 days after lipofection of the bicistronic vector pS2-S/pIL2 contained biologically active IL-2 (Fig. 2C), with most of the IL-2 accumulated in the culture supernatant. However, IL-2 activity was found only in the cellular fraction of pS2-S-IL2-transfected cells, indicating that the middle envelope and IL-2 fusion protein retained IL-2 functional activity and the pre-S2 region retained the fusion protein on the cell membrane.

Effect of cellular localization of HBV envelope proteins on antibody responses. Plasmids pS and pS2-S encoding secreted and membrane-bound HBV envelope proteins, respectively, provided us with an opportunity to study how the cellular localization of the encoded antigen might influence the magnitude of immune responses to DNA vaccines. Groups of five female C57BL/6 ($H\text{-}2^b$), C3H/HeN ($H\text{-}2^k$), and BALB/c ($H\text{-}2^d$) mice were given injections of 100 μg of plasmid DNA delivered in 100 μl of saline to the quadriceps muscles. Serum from each mouse obtained at different time intervals following injection were analyzed for the anti-HBs antibody responses. IgM anti-HBs antibodies were initially detected as early as 2 weeks after injection with either pS or pS2-S plasmids, peaked at week 4, and subsequently showed a gradual decrease in titers (Fig. 3A). A class switch to IgG antibodies was observed starting in week

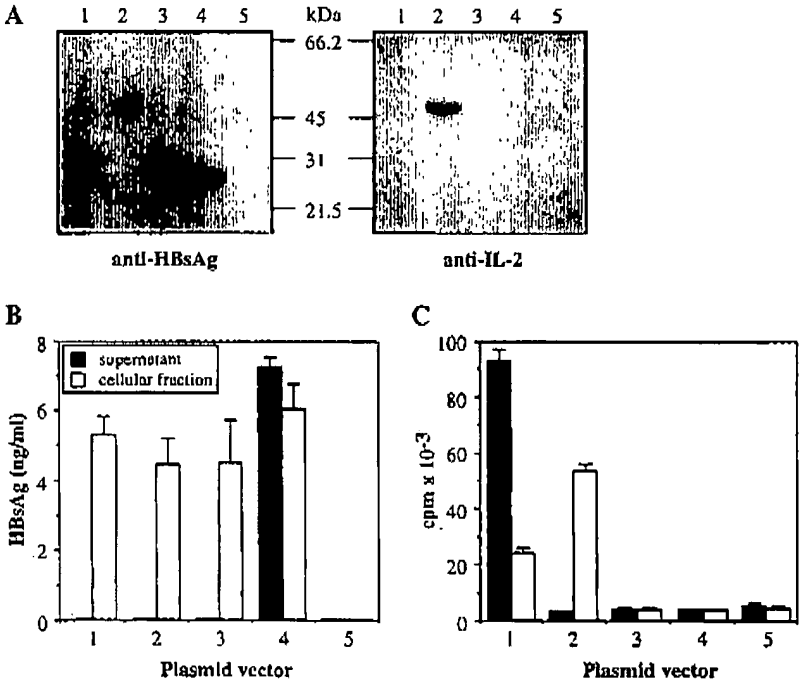

FIG. 2. Expression of HBV envelope protein and IL-2 in transiently transfected C2C12 cells. Cells were transfected with different plasmid vectors by using Lipofectamine (Gibco) as detailed in Materials and Methods. After 72 h, proteins secreted into supernatant and those remaining in the cellular fraction were prepared for analysis. (A) Immunoblot analysis. Cellular proteins of transfected cells were subjected to SDS-PAGE followed by blotting to nitrocellulose. Nitrocellulose strips were reacted with anti-HBsAg or anti-murine IL-2 antibodies and detected with HRP-conjugated second-step antibodies. (B) Amounts of HBsAg in the samples mensured by the SURASE ELISA kit (General Biologicals Co.). (C) Assay of IL-2 bioactivity. IL-2-responsive HT-2 T cells were incubated with sample proteins (diluted 1:4 in complete RPMI 1640 plus 10% FBS) from transfected cells, and proliferation was measured by [^{3}H]thymidine uptake 16 to 24 h later. Lanes: 1, pS2-S/pIL2; 2, pS2-S-IL2; 3, pS2-S; 4, pS; 5, pcDNA3.

3. Mice immunized with plasmid pS or pS2-S first revealed IgG anti-HBs antibodies at week 3, and the titers gradually increased and peaked between weeks 6 and 8 following injection (Fig. 3B). The different *H-2* genetic background had no significant effect on the antibody responses induced by HBV DNA vaccination. The cellular localization of HBV envelope proteins (secreted or membrane bound) also has no effect on the ability of DNA vaccines to elicit primary and secondary antibody responses, since mice immunized with pS2-S vector have comparable antibody titers and similar kinetics to those immunized with pS vector.

Clinically, a widely used level of protective immunity against HBV infection has been at a level of 10 mIU of anti-HBs antibodies per ml based on a World Health Organization reference preparation (7). To test whether HBV DNA vaccination can result in anti-HBs titers above that known to be protective in humans, the serum of an individual immunized mouse was analyzed with two different commercial kits: the ANTISURASE kit and the AUSAB-EIA kit. These ELISA kits determine total anti-HBs antibodies present in test sera. The AUSAB quantitation panel or a standard anti-HBs control serum provided in the kit was used to generate a standard curve to calculate total anti-HBs antibodies, which were expressed in milli-international units per milliliter. Antibody titers measured with these two kits were comparable, with differences of less than 5%. We found that 2 weeks after DNA injection, 60% of mice immunized with pS or pS2-S recombinant vectors had seroconverted to a titer of >10 mIU/ml and by week 3, all immunized mice had seroconverted (data not shown). The antibody titers gradually increased and peaked by week 8, with levels reaching between 150 and 200 mIU/ml.

Effect of coexpression of IL-2 and HBV envelope proteins on antibody responses. To establish whether the efficacy of a DNA vaccine can be modulated by simultaneous expression of IL-2, groups of C57BL/6 mice were inoculated with 100 μg of plasmid pS2-S or one of the two plasmids, pS2-S-IL2 and pS2-S/pIL2, which coexpress IL-2 activities either as membrane-bound fusion proteins or as secreted molecules. Serum samples from immunized mice were analyzed serially for 40 weeks. In all groups, antibodies to HBsAg were first detected 2 weeks after injection, and most of them at this time were of the IgM isotype (Fig. 4A). Analysis of total anti-HBs antibodies with commercial kits showed that the peak antibody titers occurred by week 8 (Fig. 4B). At 20 weeks after injection, mice immunized with plasmid pS_2-S still maintained a relatively high antibody titer (~100 mIU/ml) without further DNA injection. Coexpression of IL-2 with the antigen resulted in a much stronger IgM anti-HBs antibody response. Mice immunized with pS2-S-IL2 and pS2-S/pIL2 yielded, in general, two- to fourfold-higher IgM antibody titers from weeks 3 to 6 after vaccination compared to those of mice immunized with equivalent amounts of pS2-S (Fig. 4A). The total anti-HBs antibodies, from weeks 4 through 20 after DNA injection, were also enhanced by a factor of two- to fourfold in mice which had received plasmids coexpressing IL-2 and the envelope protein compared to those from mice immunized with pS2-S. The adjuvant effect of IL-2 subsequently decreased, until by week 32, the difference in anti-HBs titers between groups immu-

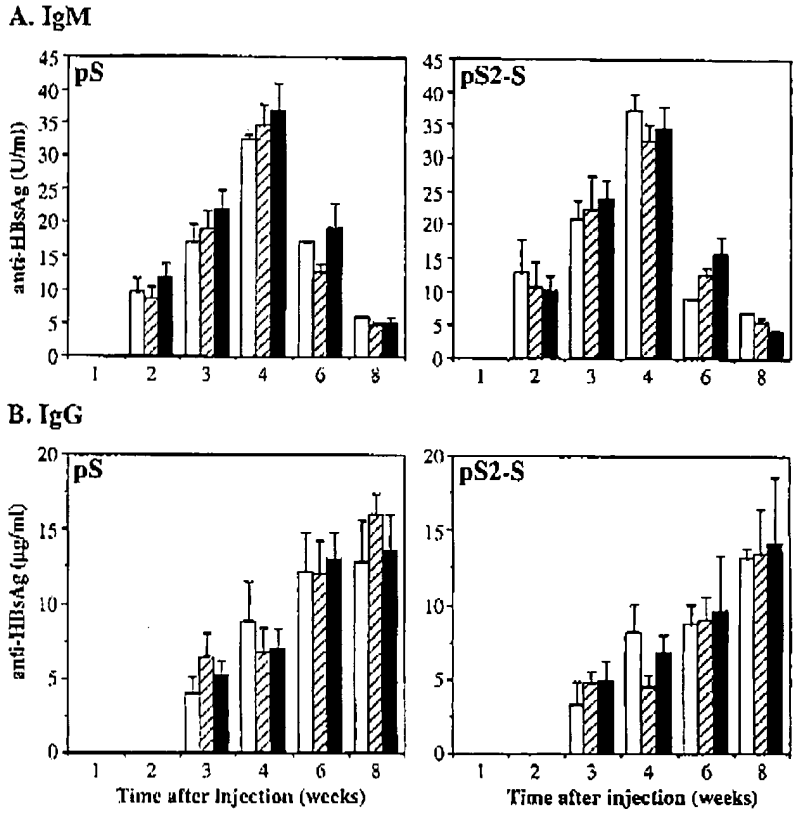

FIG. 3. Kinetics of IgM and IgG anti-HBs antibodies in mice immunized with plasmid pS or pS2-S. C57BL/6 (□), BALB/c (▨), and C3H/HeN (■) mice were given intramuscular injections of 100 μg of plasmid DNA. Sera were obtained at different time points and assayed for the presence of IgM and IgG antibodies reactive with recombinant HBsAg. Concentrations of IgM and IgG anti-HBs antibodies were measured from the standard curve generated from the respective serially diluted control antibodies and expressed as units per milliliter and micrograms per milliliter, respectively. The data are presented as mean ± SD for five animals per time point.

nized with and without IL-2 coexpression became negligible. To determine the minimal amount of DNA necessary for vaccination, the animals were given a single injection of various doses (100, 10, or 1 μg) of different HBV plasmid vectors and the anti-HBs antibody levels were assayed at weeks 8 and 20 postinjection. Figure 5 clearly shows that the antibody responses elicited by the various HBV DNA vaccines were dose dependent, with higher doses of DNA producing an apparent increase in anti-HBs antibody responses. Analysis of peak antibody titers at week 8 showed that there was a threshold dose of 10 μg for plasmids pS and pS2-S (Fig. 5A). Lower doses of these plasmids (1 μg of DNA) did not produce detectable anti-HBs antibodies in any of the immunized animals. In contrast, 1 μg of pS2-S-IL2 or pS2-S/pIL2 was effective in inducing antibody responses; the anti-HBs titers were comparable to those obtained by injection of 100 μg of pS or pS2-S. The enhancement of antibody titers by coexpression of IL-2 was demonstrated for each test dosage at weeks 8 and 20 (Fig. 5).

It has been previously reported that antibody responses after immunization with recombinant HBsAg are strongly influenced by MHC-linked genes. Mice of the $H\text{-}2^f$ haplotype have been identified as nonresponders (29, 30). To test whether plasmid DNA vaccines are effective in producing anti-HBs antibodies in a nonresponder inbred strain, groups of female B10.M ($H\text{-}2^f$) mice were given injections of 100 μg of various HBV DNA vaccines and serially bled to measure total anti-HBs titers in their sera. Mice were also immunized with a commercial yeast-derived recombinant HBsAg vaccine (H-B-VAX II) to serve as a control. The kinetics of antibody responses in B10.M mice immunized with plasmid pS2-S were similar to those in other inbred strains, with peak anti-HBs titers occurring 8 weeks after immunization (Fig. 6). However, the titer was much lower in B10.M mice (15 ± 1 mIU/ml) than in C57BL/6 (189 ± 10 mIU/ml), BALB/c (192 ± 44 mIU/ml), and C3H/HeN (204 ± 63 mIU/ml) mice, as shown above. Coexpression of IL-2 helped enhance antibody responses in B10.M mice; at week 8, the anti-HBs titers of mice immunized with pS2-S-IL2 and pS2-S/pIL2 were 51 ± 3 and 51 ± 4 mIU/ml, respectively. After the mice were boosted with the same dosage of plasmid DNA at week 14, a two- to threefold increase in antibody titers was observed in all groups of mice; at this time, anti-HBs titers in B10.M mice immunized with plasmids coexpressing IL-2 and the envelope protein were comparable to those achieved in the responder strains immunized with pS2-S. B10.M mice immunized with recombinant HBsAg vaccines did not produce any detectable anti-HBs titers (Fig. 6), whereas C57BL/6, BALB/c, and C3H/HeN mice responded well to the same treatment (data not shown).

Effect of coexpression of IL-2 and HBV envelope proteins on T-cell proliferative responses. The enhancing effect of IL-2 on Th cells was examined. Groups of C57BL/6 mice were given injections of 100 μg of different HBV plasmid vectors. At 2 weeks

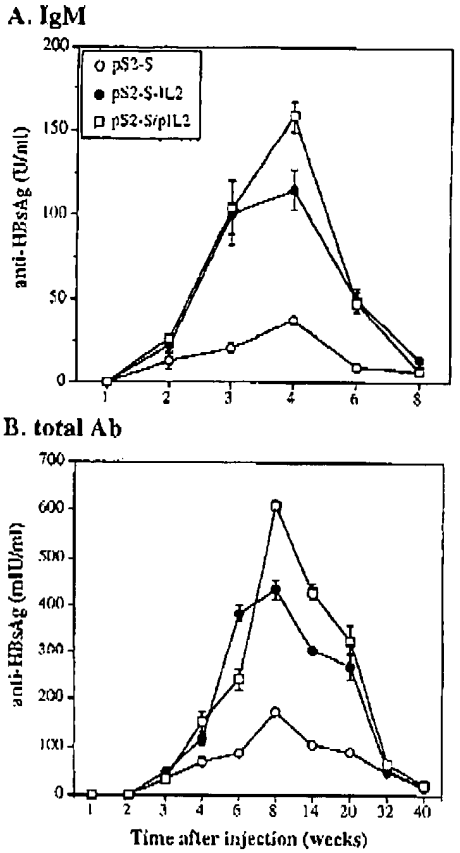

FIG. 4. Effect of coexpression of IL-2 on IgM and total anti-HBs antibodies. C57BL/6 mice were given intramuscular injections of 100 μg of plasmid pS2-S, pS2-S-IL2, or pS2-S/pIL2, and sera were collected at different time intervals and analyzed for the presence of anti-HBs antibodies. The amount of IgM anti-HBs antibody was determined as described in the legend of Fig. 3. The total amounts of anti-HBs antibodies were quantitated with a commercial kit, and values were determined relative to the standard reference preparation. Mean titers are expressed in milli-international units per milliliter. The threshold for seroconversion was defined as ≥10 mIU/ml. The data are presented as mean ± SD for five animals per time point.

after immunization, splenocytes were examined for proliferation in response to specific antigen stimulation. Splenic lymphocytes derived from pS- and pS2-S-inoculated animals demonstrated dose-dependent proliferative responses to HBsAg, with a peak stimulation index of about 7.5 for both vectors (Table 1). Coexpression of IL-2 activity by plasmids pS2-S-IL2 and pS2-S/pIL2 enhanced the cellular proliferation, with the peak stimulation index increasing to 25.4 and 26.8, respectively. All mice failed to respond to transferrin included as a control antigen. Mice vaccinated with control pcDNA3 vector did not respond to HBsAg or transferrin. Spleen cells showing proliferative responses were further tested by immunomagnetic depletion of $CD4^+$ or $CD8^+$ lymphocytes. Depletion of $CD4^+$ cells completely abolished the HBsAg-specific proliferative response, whereas depletion of $CD8^+$ cells had little effect on this response (Fig. 7). This result indicated that the bulk of the proliferative responses could be accounted for by $CD4^+$ cells. It is known that subsets of Th cells can be distinguished by the pattern of cytokines that they produce (31, 32). Th1 cells produce IFN-γ, IL-2, and lymphotoxin and play a critical role in directing cell-mediated immune responses, which are important for clearance of intracellular pathogens. Th2 cells produce IL-4, IL-5, IL-10, and IL-13; they have been associated with allergy and are important for humoral responses (35, 38, 43, 58). To distinguish between activation of Th cells of the Th1 and Th2 subsets, profiles of cytokines released by HBsAg-stimulated splenocytes were examined. Immunization of mice with plasmids encoding IL-2 in the fusion or nonfusion context resulted in a significant increase in Th1 cytokine production; IFN-γ levels were increased four- to fivefold, and IL-2 levels were increased two- to threefold (Table 1). The production of the Th2 cytokine IL-4 by stimulated splenocytes was also increased in animals inoculated with plasmids coexpressing IL-2 and the envelope protein compared with those immunized with pS2-S, although the difference was much less significant. These results indicated that coexpression of IL-2 with the envelope protein by HBV DNA vaccines enhanced mainly Th cells of the Th1 subsets. We then investigated the dose-response relationships between doses of different HBV DNA vaccines and Th-cell proliferative responses. Similar to their antibody response, Th-cell responses were also

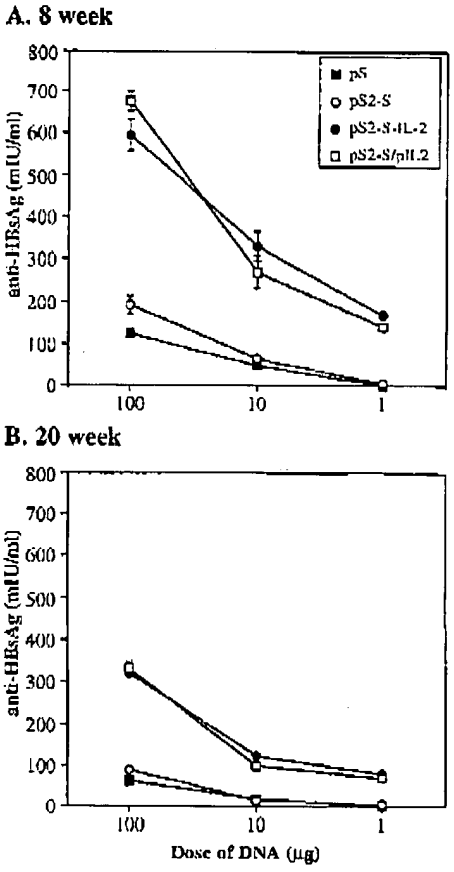

FIG. 5. Effect of vaccine dosage and coexpression of IL-2 on anti-HBs antibodies. C57BL/6 mice were given intramuscular injections of 100, 10, or 1 μg of plasmid pS2-S, pS2-S-IL2, or pS2-S/pIL2. Sera were collected 8 and 20 weeks after immunization and analyzed for the presence of anti-HBs antibodies with a commercial ELISA kit as detailed in the legend to Fig. 4. The data are presented as mean ± SD for five animals per time point.

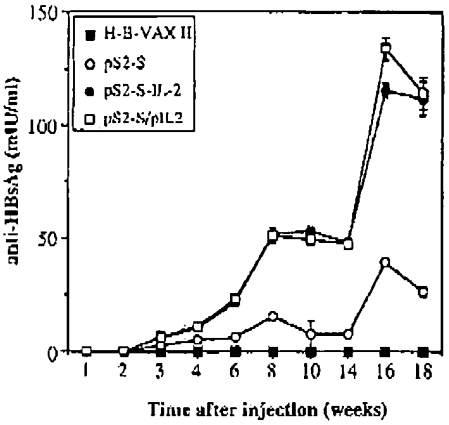

FIG. 6. Effect of coexpression of IL-2 on anti-HBs antibodies in HBsAg nonresponder mice. Groups of B10.M ($IL\text{-}2^{r}$) mice were given intramuscular injections of with 100 μg of plasmid pS2-S, pS2-S-IL2, or pS2-S/pIL2 and boosted with the same amount of DNA 14 weeks later. As a control, one group of mice was immunized with 4 μg of yeast-derived recombinant HBsAg vaccine (H-B-VAX II) and boosted with the same dose 3 and 14 weeks later. Sera were collected at different time points and analyzed for the presence of anti-HBs antibodies. The data are presented as mean ± SD for five animals per time point.

dose dependent in all groups of mice. Plasmids coexpressing IL-2 activity were much more effective in inducing Th-cell responses than were plasmids encoding HBsAg alone. As little as 1 μg of pS2-S-IL-2 or pS2-S/pIL2 was sufficient to induce significant Th-cell responses comparable to those induced by 100 μg of plasmid pS2-S (Fig. 8).

DISCUSSION

In this study, we have constructed plasmid expression vectors encoding various forms of HBV envelope proteins and have shown that animals vaccinated with these plasmid vectors develop long-lasting immunity against HBsAg. The cellular localization of the encoded antigen, whether secreted or anchored on the membrane of transfected cells, has little effect on the efficacy of DNA vaccines. By contrast, we have shown that coexpression of IL-2 and the HBV envelope protein within the same plasmid vector resulted in at least a 100-fold increase in its ability to induce humoral and cellular immune responses to HBsAg.

Since the introduction of the genetic vaccination approach in 1993 (45), many groups have successfully applied this technique to develop DNA vaccines against infectious pathogens in many different animal models (21–24, 28, 40, 41, 45, 46, 53, 56, 57); however, the efficacy of different DNA vaccines has varied widely. Some DNA vaccines are incapable of inducing specific immune responses even after several inoculations of large amounts of plasmid DNA (25, 34, 55, 57). The low responsiveness to some viral antigens, such as the nucleoprotein of lymphocytic choriomeningitis virus, observed in some DNA vaccines cannot be ascribed to the inherent low immunogenicity of the antigen, since the same antigen expressed by viruses has been shown to induce strong nucleoprotein-specific antibody responses (57). Another explanation for the suboptimal immune responses may be related to the cytoplasmic localization of the nucleoprotein in the transfected cells. It has been shown that muscle cells are the major targets of the transfected genes after plasmid DNA inoculation (1, 45, 50). However, myoblasts and myocytes express only low levels of MHC class I determinants and appear not to express MHC class II and the accessory costimulatory molecules, such as B7-1/B7-2 (17), which are important for Th-cell activation (4, 19). Thus, it is unlikely that the transfected myoblasts/myocytes serve as antigen-presenting cells to stimulate $CD4^{+}$ Th lymphocytes. A possible pathway for priming T cells may involve the release of the encoded antigen from the transfected muscle cells to the draining lymph nodes. There, these antigens could be recognized by B cells and could be processed by "professional" antigen-presenting cells, such as macrophages and dendritic cells, for presentation to T cells with appropriate costimulation. If this mechanism exists, the plasmid vector encoding a secreted viral antigen is likely to be more efficacious as a DNA vaccine. To test this hypothesis, we used two HBV plasmid constructs, pS and pS2-S. Plasmid pS encodes the major envelope protein, which is secreted to the culture supernatant from transfected cells. By contrast, the pS2-S-encoded middle envelope protein, which contains the 55-amino-acid pre-S2 region in addition to the S protein, is expressed mainly on the cell membrane. We found that animals of different MHC haplotypes, when immunized with plasmids pS and pS2-S, developed antibody responses similar in time course and magnitude despite the different localization of the encoded antigens (Fig. 3). There are also no significant differences in T-cell proliferative responses induced by these two vectors (Table 1). Similar results were reported by Xiang et al. (54), who found that immune responses induced by DNA vaccines expressing secreted or membrane-bound rabies virus G proteins were similar in magnitude. These results suggest that a DNA vaccine expressing a secreted protein has no advantage over a DNA vaccine expressing a membrane-bound protein. A possible mechanism for the membrane-bound protein to induce immune responses is that due to the inoculation of the plasmid DNA, some

TABLE 1. HBsAg-specific T-cell responses to vaccination with different HBV plasmid vectors[a]

Vector	T-cell stimulation index with stimulant[b]:			Amt of cytokine released (pg/ml)[c]:		
	HBsAg		Transferrin (120 μg/ml)	IFN-γ	IL-2	IL-4
	30 μg/ml	10 μg/ml				
pcDNA3	1.4 ± 0.4	1.2 ± 0.3	1.0 ± 0.2	41 ± 0	103 ± 0	38 ± 0
pS	7.4 ± 0.4	4.5 ± 0.7	1.7 ± 0.4	402 ± 13	430 ± 7	142 ± 27
pS2-S	7.5 ± 0	3.8 ± 0.6	1.2 ± 0.8	477 ± 5	456 ± 8	205 ± 6
pS2-S-IL2	25.4 ± 3.2	12.3 ± 1.2	1.0 ± 0.2	1,734 ± 77	1,169 ± 32	329 ± 15
pS2-S/pIL2	26.8 ± 3.6	13.4 ± 0.6	1.4 ± 0.5	2,122 ± 50	1,330 ± 59	271 ± 23

[a] C57BL/6 mice were given intramuscular injections of 100 μg of different HBV plasmid vectors. Splenocytes pooled from three immunized mice were used in proliferation assays. Culture supernatants obtained 3 days after stimulation were collected for quantitation of IFN-γ, IL-2, and IL-4 by ELISA.
[b] Values are mean stimulation index for triplicate wells ±SD.
[c] Values are mean concentration for triplicate wells ±SD.

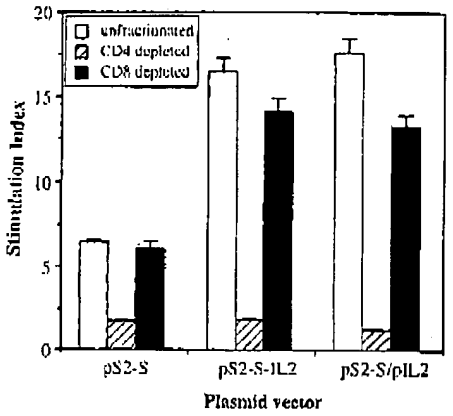

FIG. 7. Lymphocyte proliferative responses to HBsAg following depletion of spleen cells from mice immunized with different HBV plasmid vectors by using anti-murine $CD4^+$ or $CD8^+$ antibodies. C57BL/6 mice were given intramuscular injections of 100 μg of plasmid pS2-S, pS2-S-IL2 or pS2-S/pIL2. Splenocytes pooled from three immunized mice were used in proliferative assays. The preparation of CD4-depleted and CD8-depleted splenocytes is detailed in Materials and Methods. Values are presented as the mean stimulation index for triplicate wells ± SD.

damaged muscle fibers release a small quantity of antigens to reach the draining lymph nodes. There, the antigens are recognized by B cells and other antigen-presenting cells and function in antigen processing and presentation to activate naive Th cells. Thereafter, the prolonged cell surface expression of antigens and the MHC class I/peptide complex display may serve to boost activated B cells and T cells, respectively, which are considerably less dependent on accessory cell costimulation than are naive cells. This hypothesis was supported by our finding that the membrane-bound IL-2 was as effective as the secreted form of IL-2 in enhancing immune responses to HBV DNA vaccines (Fig. 4, 5, and 8).

A large number of cytokines, such as IL-1β, IL-2, and IFN-γ, have been shown to be capable of enhancing various immune responses when administered for prolonged periods during the development of an immune response (8, 15). The adjuvant effects of cytokines were also observed in the case of genetic immunization. Plasmid vectors containing a CpG dinucleotide motif elicited much stronger humoral and cellular immune responses to the encoded antigens than did vectors which did not contain this sequence (39). The adjuvant activity of the CpG motif was closely related to its ability to elicit a rapid cytokine release from the transfected cells. A more direct evidence that cytokines could influence the efficacy of DNA vaccination was shown by Irvine et al. (18). These authors showed that in a mouse tumor model, when recombinant IL-2, IL-6, IL-7, or IL-12 was added following administration of DNA encoding a tumor-associated antigen, the number of established metastases was significantly reduced compared with that in mice treated with DNA only. However, because of the pleiotropic nature of cytokines, the systemic administration of cytokines at the therapeutic levels produced not only the intended immune induction but also undesirable nonspecific responses. A sustained but low level of cytokines delivered to tissues of immune interactions may reduce the toxicity of these pleiotropic compounds while improving their therapeutic and practical value in providing vaccine adjuvant effects. Direct injection into mouse skeletal muscle of expression vectors encoding cytokines provides such a means. Raz et al. (36) reported that intramuscular injections of plasmids encoding IL-2, IL-4, or type β1 transforming growth factor successfully modulate immune responses to transferrin delivered at a separate site. Plasmid encoding IL-2 enhanced both cellular and humoral immune responses, while plasmid encoding type β1 transforming growth factor depressed the anti-transferrin response. Injection of a cDNA encoding IL-4 into muscle selectively increased IgG1 levels but did not alter the cellular response. It was also reported that coinjection of plasmids encoding IL-2 (47) or granulocyte-macrophage colony-stimulating factor (GM-CSF) but not IFN-γ (52) enhanced immune responses to the DNA vaccines. The enhancing effect of the GM-CSF-expressing vector was dose dependent; greater than 50 μg of DNA was required to cause an observable adjuvant effect, and the maximum adjuvant effect was not achieved until a dosage of 250 μg. The adjuvant effect of GM-CSF-expressing vector was also dependent on coinjection with the plasmid encoding viral antigen; inoculation of the two plasmids separately several hours apart had no effect on the magnitude of specific antibody response, indicating that colocalization of cytokines and viral antigens at the site of immune interaction may be important for the cytokine adjuvant effects. In our studies, we have used the same plasmid vector to encode both HBV envelope protein and IL-2 in the fusion or nonfusion context to ensure that both proteins are synthesized by the same transfected cells and thus may reach antigen-presenting cells together. We showed that coexpression of IL-2 not only enhanced the cellular and humoral anti-HBs immune responses but also significantly increased the efficacy of DNA vaccines. As little as 1 μg of plasmid pS2-S-IL2 or pS2-S/pIL2 was sufficient to induce significant antibody titers and Th-cell responses comparable to those achieved by 100 μg of plasmid expressing the envelope protein alone. This is much more efficient than immunization with antigens and cytokines expressed by two different plasmid vectors as reported by Xiang et al. (52).

The protective value of the immune response is highly dependent on the types of cytokines produced by T cells. Th1 cells, producing IL-2, IFN-γ, and lymphotoxin, are potent enhancers of cell-mediated immune responses and play a critical role in the clearance of intracellular pathogens, while Th2

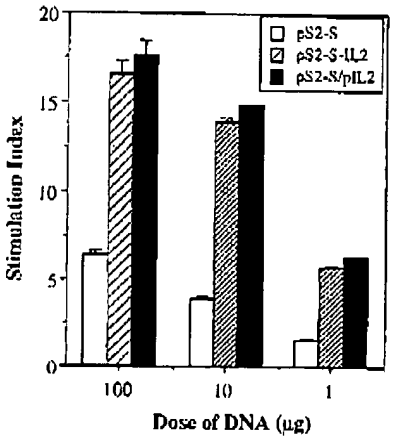

FIG. 8. Effect of vaccine dosage and coexpression of IL-2 on HBsAg-specific lymphocyte proliferative responses. C57BL/6 mice were given intramuscular injections of 100, 10, or 1 μg of plasmid pS2-S, pS2-S-IL2, or pS2-S/pIL2. Splenocytes pooled from three immunized mice were used in proliferative assays. Values are presented as the mean stimulation index for triplicate wells ± SD.

cells, which produce mainly IL-4, IL-5, and IL-10, are important for antibody responses (35, 38, 43, 58). In the *Leishmania* system, Th1 cells give protection while Th2 cells cause susceptibility (16, 43). Several groups have demonstrated that cytokines themselves have striking effects on the differentiation and/or growth of T cells that express different cytokine-producing phenotypes (42). Th2 cells develop when naive T cells are stimulated in vitro in the presence of IL-4. Conversely, IL-12 is a critical factor driving the development of Th1 cells from antigen-specific naive $CD4^+$ T cells. IL-2 is an important T-cell growth factor and appears to be required for naive cells to develop into Th1 or Th2 cells but is not so important in their differentiation fate. It has been reported that IL-2, when given in repeated doses as an adjuvant, tends to enhance Th1- but not Th2-like responses (33, 49). Our results with plasmid vectors coexpressing HBV envelope protein and IL-2 showed that both antibodies and cellular proliferative responses were enhanced, although the increase of Th1-like responses is much more significant than that of Th2-like responses in this reaction.

Immunization with vaccines containing HBsAg, prepared from plasma or by genetic engineering techniques, has been effective in preventing the establishment of chronic HBV infections (10). However, approximately 14% of vaccinees remain only mildly responsive to nonresponsive after three doses of vaccine (2). Studies have shown that the nonresponsiveness to the HBsAg vaccine is related to certain MHC haplotypes (2, 6). Application of IL-2 with antigens during immunization has been reported to overcome MHC-linked nonresponsiveness to peptide antigens (12, 20) or to reverse the nonresponsiveness to HBsAg vaccination in immunocompromised patients (27). We have shown in this paper that coexpression of IL-2 dramatically increases the efficacy of HBV DNA vaccines. Moreover, the IL-2 adjuvant activity helps elicit high anti-HBs titers in animals (B10.M; $H\text{-}2^f$) which usually fail to respond to recombinant HBsAg vaccination (29, 30). We believe that the use of DNA vaccines in combination with IL-2 adjuvant may provide a simple approach to overcome the nonresponsiveness to HBsAg vaccines as well as vaccines for a variety of other diseases.

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